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Bacterial Persistence and Immunity in Goats Vaccinated with a *purE* Deletion Mutant or the Parental 16M Strain of *Brucella melitensis*

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To evaluate host responses, young goats were inoculated subcutaneously with a genetic deletion mutant (Δ*purE201*) of *Brucella melitensis* ($n = 6$), its virulent parental strain 16M ($n = 6$), or saline ($n = 6$). No clinical evidence of brucellosis was seen in any goat. Serum antibody titers peaked at postinoculation day (PID) 14. Bacteria in lymph nodes that drained sites of vaccination reached peak numbers of $>10^6$ CFU/g in both infected groups at PID 7 and progressively declined to PID 84. At necropsy, bacteria were present in mammary lymph nodes or spleen of 33% of goats given virulent 16M but in none of goats given the *purE* mutant. Lymphadenitis, most severe in goats given 16M, involved depletion of lymphocytes and germinal centers, proliferation of lymphoblasts, and vasculitis. By PID 28, lymph node architecture was restored; there was marked germinal center formation and medullary plasmacytosis. Brucellar antigens, detected with immunoperoxidase techniques, were prominent in capsular granulomas but not in lymph node cortices. Ultrastructurally, bacteria were found in macrophages (>97%) and small lymphocytes (<3%) but not in large lymphocytes. Bacteria were intact in small lymphocytes but in macrophages were in various stages of degradation. The Δ*purE* phenotype of Δ*purE201* was preserved during infection of goat lymph nodes. Unlike *Salmonella* spp. *purE* mutants, strain Δ*purE201* may be a candidate for efficacy testing; it produced immune responses, was cleared from visceral tissues, and produced less severe pathologic changes than its wild-type parent.

Live mutant strains of *Brucella* spp. derived by classic selection methods are widely used as vaccines in animals; e.g., *Brucella abortus* 19 and RB51 are used in cattle, and *B. melitensis* Rev 1 is used in sheep and goats. Recent vaccine strategies for brucellosis have included deletion of specific genes in order to develop candidate vaccine strains with reduced virulence (4, 13, 27, 29). A *purE* gene deletion mutant (Δ*purE201*) of *B. melitensis* was constructed as a potentially useful vaccine for humans and animals (9, 15). In contrast to the parental 16M strain, Δ*purE201* fails to grow in cultured human monocyte-derived macrophages and has shown reduced virulence in mice (9).

Because of safety concerns on the use of genetically altered microorganisms, candidate vaccine strains should be screened for unusual biological behavior in small numbers of animals prior to large-scale testing for efficacy. *B. melitensis* mutants must be tested in goats, the natural hosts (17, 20). Although the goat has been widely used as a model for *B. abortus*-induced brucellosis (1, 21), the pathogenesis of *B. melitensis* in goats has not been adequately examined.

This biosafety study was initiated to analyze clinical, immunologic, and pathologic responses of goats to large numbers of bacteria, i.e., to determine a maximum dose that would be considered reasonable in field use. The experiment was designed to acquire data on clearance of bacteria from lymph nodes that drain sites of vaccination, on immune responses, and on the development of untoward pathologic manifestations of infection. It is important that visceral organs and central nervous system of animals given genetically engineered mutants of *B. melitensis* be free of vaccine-induced pathologic change. Our hypothesis is that the Δ*purE201* mutant will persist in host lymph node tissue for time sufficient to induce immunity but insufficient to be reactivated at sexual maturity.

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MATERIALS AND METHODS

Experimental design. *B. melitensis* 16M, the parent of Δ*purE201*, was obtained from Gerhardt Schurig, Virginia Polytechnic University. Strain 16M retained its virulence in mice and human monocyte cultures (9). Δ*purE201* was constructed by chromosomal gene replacement (9, 15). The resulting kanamycin-resistant organism did not grow on minimal medium but grew if the medium was supplemented with purines. Compared with its parent, Δ*purE201* was attenuated for growth in human monocyte-derived macrophages (9). Eighteen young goats were inoculated subcutaneously with one of three suspensions: *B. melitensis* 16M ($n = 6$), *B. melitensis* Δ*purE201* ($n = 6$), and saline ($n = 6$). Four-month-old goats were obtained from one brucellosis-free herd and housed in groups of six in separate pens in an isolation building certified for biosafety level 3 biocontainment, i.e., with air pressures that were positive to an exterior filtration system and negative to a clean vestibule with HEPA-filtered exterior air.

Goats were injected subcutaneously with *B. melitensis* or saline in the axillary region that drains into the superficial cervical lymph node. The dose per goat was 16.08×10^9 CFU for the 16M group and 16.16×10^9 for the Δ*purE201* mutant group; each dose was split and given at two sites, half on the right and half on the left (approximately 8×10^9 CFU in 2 ml at each site). Goats were examined daily for evidence of disease. Postmortem examinations were done on all goats at postinfection day (PID) 116, the end of the experiment. Tissues fixed in 10% buffered formalin for histopathologic examination included bone marrow, brain, joint, kidney, liver, lung, lymph nodes (parotid, supramammary, iliac, and prefemoral), mammary gland, spleen, and uterus. Blood and cerebrospinal fluid were collected and frozen.

The left superficial cervical lymph node was biopsied from two goats in each

group on PID 3, 7, and 14. The right superficial cervical lymph node was biopsied from two goats in each group on PID 28, 56, and 84 (4). For bacterial culture, approximately one-fifth of the lymph node mass was sampled at each of three sites: distal and proximal ends, and center of the node. Remaining tissue was placed in medium for blastogenesis assay and fixed in 10% formalin or 2.5% glutaraldehyde in cacodylate buffer. Paraffin sections were stained with standard hematoxylin-eosin and with an avidin-biotin-peroxidase complex immunoenzyme technique (20, 21).

Bacteriology. Brucellae for vaccination were grown on tryptose agar at 37°C for 3 days (4). Bacteria were cultured from tissue onto tryptose agar with 5% bovine serum or tryptose agar with 5% bovine serum, cycloheximide (30 μg/ml), bacitracin (7.5 U/ml), polymyxin B sulfate (1.8 U/ml), and ethylviolet (1:300,000). Blood (10 ml) from each goat was added to tryptose broth (10 ml) containing 1% sodium citrate; bacteremia was examined on PID 3 and 116 by using 20 ml of peripheral blood.

Randomly selected *B. melitensis* Δ*purE201* colonies (n = 89) isolated from blood and lymph nodes of infected goats were plated onto M9 minimal agar, M9-adenine agar, brucella agar, and brucella agar with 50 μg of kanamycin per ml. M9 medium was supplemented with 0.2 g of Casamino Acids, 0.1 mM tryptophan, 0.05 mM thiamine, and 0.2 g of glucose. Purine-containing M9 plates were made by supplementing the medium with 5.0 mM adenine.

Chromosomal DNA was isolated (14) from 6 of the 89 *B. melitensis* colonies. The concentration of DNA was determined by spectrophotometry and adjusted to 100 μg/ml. Chromosomal DNA (200 ng) was digested with *Eco*RI (Boehringer Mannheim Biochemicals) for 2 h at 37°C. The DNA fragments were separated by electrophoresis in a 1.2% agarose gel in 1× Tris-acetate-EDTA buffer, transferred to Nytran membranes, UV cross-linked, and hybridized with a digoxigenin-labeled probe (Boehringer Mannheim Biochemicals). The probe was prepared by PCR amplification of the *purE* gene with forward primer ATC GCA CCC CTG ACA GGC T and reverse primer GCC GCT GCC ATG GCG AG, which flank the deletion of *purE* carrying the kanamycin cassette (9).

Immunologic testing. Lymphocyte blastogenesis in response to brucellar antigens was determined by isolating lymphocytes from lymph node biopsy specimens (28). Killed suspensions of *B. melitensis* 16M and Δ*purE201* were prepared by gamma irradiation. Cells were harvested onto glass filter mats and counted for radioactivity in a liquid scintillation counter. Cell proliferation results were expressed as mean counts per minute ± standard deviation. Dot blot assays of Δ*purE201* on goat sera were used to determine antibody titers (4). Suspensions of *B. melitensis* 16M and Δ*purE201*, killed by gamma irradiation, were adjusted to 20% transmission at 600 nm; 30 μl of suspension was added to each test well. Tenfold dilutions of serum (30 μl) were used. Horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin G (IgG; Jackson Immunoresearch Laboratories, West Grove, Pa.) was diluted 1:15,000, and 100 μl was added to all wells. Results were expressed as the highest serum titer that produced a visible color reaction on nitrocellulose that was greater than the reaction for a negative serum sample.

Statistical analyses. Analysis of variance of bacteria numbers (CFU per gram) in superficial cervical lymph nodes was tested in a split-plot model with use of SAS software. In this analysis, we assume that the removal of one lymph node would not change the bacterial numbers in the remaining lymph node. This model takes into account the correlation in bacterial counts of the three zones of the lymph nodes. For effects that showed significance of $P < 0.05$, Tukey's Studentized range tested the differences in means. For statistical comparison of the antibody titers determined by dot blot assay, the titer was transformed to $\log_{10}$ and examined by a repeated measure design. Differences in means of group × PID were checked by Tukey's Studentized range.

RESULTS

Clinicopathologic evaluation. All infected goats developed a transient 48-h period of slight fever and anorexia after subcutaneous inoculation of *B. melitensis* 16M and Δ*purE201*. Clinically, swelling of superficial cervical lymph nodes occurred at PID 3 and was most severe at PID 7, when palpable nodes of the 16M group (ranges, 28 to 29 mm [width] and 57 to 64 mm [length]) exceeded those of the Δ*purE201* group (ranges, 15 to 21 mm [width] and 27 to 56 mm [length]), and both were larger than lymph nodes of control goats (ranges, 11 to 12 mm [width] and 17 to 21 mm [length]). At necropsy, all tissues of infected and control goats were grossly and microscopically normal; lymph nodes of infected goats were similar in size and structure to those of control goats. The presence of ovarian follicles and a uterine diameter of >2.5 cm at necropsy indicated that most goats had begun sexual development.

Bacteriology. Bacteriologic culture of superficial cervical lymph nodes from goats inoculated with strains 16M and Δ*purE201* yielded large numbers of bacteria at PID 3. Numbers of bacteria per gram of lymph node had increased at PID 7 and, although remaining high, significantly decreased (α = 0.05) at PID 14 and 28 (Table 1). The CFU per gram of lymph node tissue varied markedly in the three sites within individual lymph nodes (standard error of the mean = 2.58×10^6). The level in proximal tissue (mean = 1.17×10^6 CFU/g) was significantly lower than that in tissue from central (mean = 6.66×10^6 CFU/g) or distal (mean = 7.51×10^6 CFU/g) sites. *B. melitensis* was isolated from the bloodstream at PID 1 and 2. Bacteremia on PID 3 was found in three goats given 16M (goats 2, 3, and 4) and three goats given Δ*purE201* (goats 7, 8, and 11). Bacteremia was not detected on PID 116. All tissues taken at necropsy from Δ*purE201*-infected and control goats were free of *B. melitensis*. Tissues of 16M-infected goats were free of *B. melitensis* at necropsy except for the supramammary lymph nodes of goat 1 and spleen of goat 3 (1.06×10^1 CFU/g).

B. melitensis Δ*purE201* did not change genetically during replication in lymph node tissue of goats; 89 randomly selected colonies of bacteria isolated from tissues of Δ*purE201*-infected goats were cultured for 1 week on *Brucella* agar plates with kanamycin and on minimal agar plates. All colonies examined retained the phenotype of strain Δ*purE201* (failure to grow on minimal medium but resistance to kanamycin and growth on purine-supplemented medium).

To determine the genetic stability of *B. melitensis* Δ*purE201*, we tested colonies isolated from tissues of infected goats for purine auxotrophy and kanamycin resistance. All 89 independently isolated *B. melitensis* Δ*purE201* colonies from randomly selected blood and lymph node samples required purines for growth and were kanamycin resistant. The *purE* probe hybridized to an *Eco*RI fragment of 1.1 kb (Fig. 1) for six of the independent colonies isolated from goats (lanes 5 to 10) and to *B. melitensis* Δ*purE201* (lane 4). The probe hybridized to a 7-kb *Eco*RI fragment of *B. melitensis* 16M (lanes 3 and 11). The additional bands detected by the probe in lane 5 were the result of partial digestion by *Eco*RI of the genomic DNA from this isolate. In other experiments, complete digestion eliminated these bands (data not shown). These results show that the *purE* mutation of *B. melitensis* 16M was stable and that the vaccine could be differentiated from wild-type strains by hybridization, purine auxotrophy, and kanamycin resistance.

Immunology. All goats in this study were free of serologic evidence of infection with *B. melitensis* before vaccination. After vaccination, antibody responses and cutaneous reactivity to brucellin developed in all infected goats. Goats that received *B. melitensis* 16M had slightly higher serological responses than goats that received Δ*purE201*. In the standard tube test using antigens of *B. melitensis*, all goats had high antibody titers of 800 at 2 weeks postinfection; these titers progressively declined but were still present at the termination of the experiment at PID 120 to 134 (Fig. 2).

Dot blot titers for goats vaccinated with 16M peaked at PID 14 to 28 and progressively declined but were still detectable at PID 112 to 116 (Table 2). Titers of goats vaccinated with Δ*purE201* peaked at PID 28 and were also detectable at necropsy. Goats vaccinated with 16M had higher titers in the dot blot assay than Δ*purE201* vaccinates throughout the study.

Lymph node cells from biopsies of both groups of vaccinated goats first had proliferative responses to gamma-irradiated *B. melitensis* strain 16M or Δ*purE201* at 12 weeks postinfection, and responses were still present at 16 weeks postinfection (Fig. 3). Cells from Δ*purE201* vaccinates tended to show greater proliferative responses to strain 16M or Δ*purE201* at 16 weeks compared with responses at 12 weeks. Lymph node cells from

TABLE 1. Number of live *B. melitensis* per gram in three areas of superficial cervical lymph nodes

PID	Group	Goat	N CFU of *B. melitensis*/g[a]			
			Mean	In three zones of lymph nodes		
				Proximal	Central	Distal
3	16M	1	4.95×10^6	2.96×10^6	4.33×10^6	4.77×10^6
		2		9.12×10^5	6.81×10^6	9.91×10^6
	Δ*purE201*	7	1.06×10^7	7.99×10^5	1.84×10^6	3.26×10^6
		8		1.00×10^7	2.51×10^7	2.26×10^7
7	16M	3	1.29×10^7	1.81×10^6	1.41×10^7	1.67×10^7
		4		2.21×10^6	1.93×10^7	2.31×10^7
	Δ*purE201*	9	2.31×10^7	3.91×10^6	4.53×10^7	4.64×10^7
		10		9.26×10^5	1.32×10^7	2.88×10^7
14	16M	5	2.32×10^6	7.78×10^5	4.40×10^5	1.36×10^6
		6		1.55×10^6	2.85×10^6	6.97×10^6
	Δ*purE201*	11	3.38×10^6	9.90×10^4	2.27×10^6	1.03×10^7
		12		1.71×10^5	2.39×10^6	5.04×10^6
28	16M	1	2.03×10^5	3.50×10^4	3.59×10^4	5.86×10^4
		2		3.27×10^5	1.54×10^5	6.06×10^5
	Δ*purE201*	7	6.99×10^4	4.84×10^4	6.36×10^4	1.57×10^5
		8		1.80×10^4	3.70×10^4	9.54×10^4
56	16M	3	2.43×10^2	1.26×10^2	7.33×10^1	5.29×10^2
		4		6.04×10^2	1.20×10^2	6.86
	Δ*purE201*	9	2.97×10^2	6.08×10^1	6.89×10^1	1.75×10^1
		10		3.96×10^2	4.66×10^2	7.72×10^2
84	16M	5	1.87×10^1	0	0	8.23×10^1
		6		0	3.01×10^1	0
	Δ*purE201*	11	2.85	1.71×10^1	0	0
		12		0	0	0

[a] Means are derived from six tissue samples (proximal, middle, and distal one-fifth of one lymph node from two goats) at each time point. Standard error of the mean = 4.04×10^6. All tissues from control goats had values of 0.

nonvaccinated goats did not show proliferative responses to gamma-irradiated *B. melitensis* strains in any sampling period.

Pathology of regional lymph nodes. The initial response of superficial cervical lymph nodes at PID 3 to *B. melitensis* Δ*purE201* was acute, diffuse lymphadenitis; at this time, the dominant pathologic changes in cortical lymphoid tissues were cell death of small lymphocytes, proliferation of large lymphoblasts, and vasculitis (Fig. 4). Lymphadenitis was most severe at PID 7 and progressively declined, with a return to normal lymph node architecture at PID 28. Lymph node components that were most affected by inflammation were capsular vascular and lymphatic beds, subcapsular and peritrabecular sinusoids, and deep cortex of the lymph node.

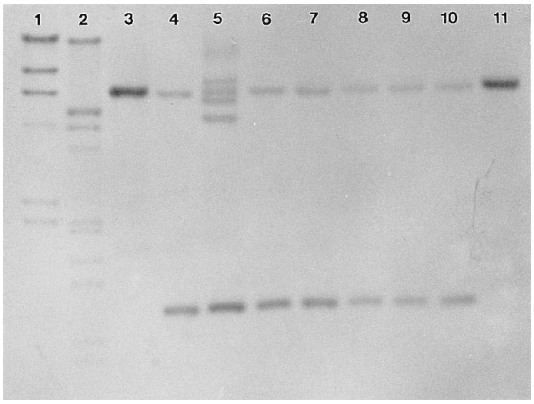


FIG. 1. Hybridization of *B. melitensis* DNA with a *purE* probe. Genomic DNA from *B. melitensis* 16M (lanes 3 and 11), *B. melitensis* Δ*purE201* (lane 4), and *B. melitensis* isolated from goats infected with Δ*purE201* (lanes 5 to 10) was digested with *Eco*RI and separated by electrophoresis through 1.2% agarose in 1× Tris-acetate-EDTA buffer. Fragments were transferred to a nylon membrane and probed with *purE* DNA. Digoxigenin-labeled molecular weight standards (Boehringer Mannheim II and III) were run in lanes 1 and 2. The additional bands in lane 5 are the result of partial digestion by *Eco*RI of the genomic DNA of this isolate.

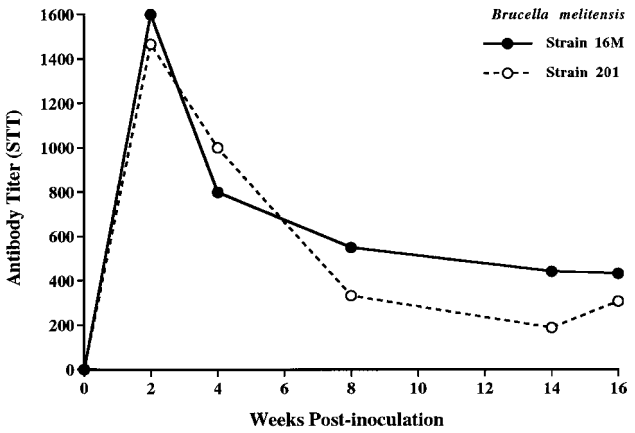


FIG. 2. Serum antibody responses of goats inoculated subcutaneously with *B. melitensis* Δ*purE201* (Strain 201) or 16M as measured by standard tube agglutination tests (STT) using *B. melitensis* tube antigen.

Differences in lymph node responses of the two infected groups were apparent at PID 7, when 16M-infected goats had severe paracortical lymphocyte depletion, destruction of germinal centers, and serofibrinous lymphadenitis with effacement of lymph node architecture (Fig. 5). The Δ*purE201*-infected goats had greater blast cell mitosis, proliferation of large lymphocytes, and preservation of germinal centers and lymph node architecture.

Capsules of affected lymph nodes were inflamed and contained foci of necrosis, microabscesses, and microgranulomas. Capsular tissue was expanded by dilatation of lymphatics, dif-

TABLE 2. Antibody titers determined by dot blot assays[a]

Goat	Titer on PID:				
	14	28	56	84	112–116
Group 16M					
1	3.71	3.41	3.41	3.11	3.11
2	3.71	3.41	3.41	3.11	3.11
3	3.71	3.71	3.11	3.11	3.41
4	2.08	4.01	4.01	4.01	4.01
5	3.41	3.41	3.11	2.81	2.81
6	3.11	3.71	3.11	3.41	3.11
Mean	3.29	3.61	3.36	3.26	3.26
SEM	0.24	0.09	0.13	0.15	0.15
Group Δ*purE201*					
7	3.11	3.41	3.11	3.11	2.81
8	3.11	3.11	2.81	3.11	2.51
9	2.81	2.81	2.51	1.90	1.60
10	2.81	3.71	3.11	2.81	2.81
11	2.81	3.11	3.11	2.81	2.81
12	3.41	3.11	2.51	2.51	2.20
Mean	3.01	3.21	2.86	2.71	2.46
SEM	0.22	0.11	0.011	0.10	0.18

[a] The control group did not have evidence of antibody. Titers are $\log_{10}$ transformed. Significant differences occurred between groups on days 28, 56, 84, and 112.

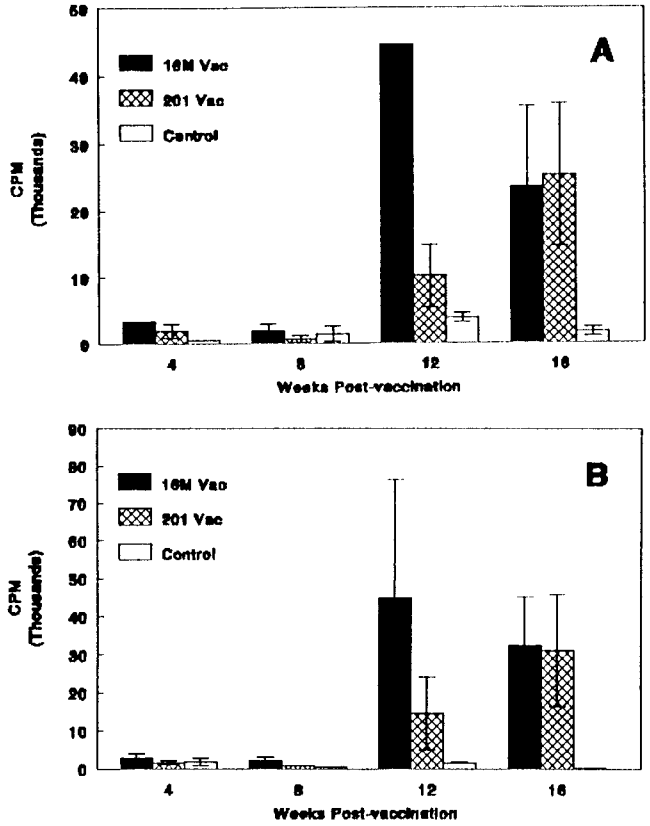


FIG. 3. Lymphocyte blastogenesis responses by vaccinated (Vac) and control goats to gamma-irradiated *B. melitensis* 16M (A) or Δ*purE201* (201) (B). Lymph node cells were incubated for 7 days with 10^6 bacteria per well. Results are expressed as mean ± standard deviation of two goats per treatment per sampling time.

fuse granulomatous lesions, marked edema, and deposition of ground substance and collagen. Subcapsular and peritrabecular sinusoids were dilated and contained large numbers of monocytes and macrophages with ultrastructural evidence of activation but were not affected by exudative changes or necrosis.

Large lymphoblasts were increased markedly and replaced sites depleted of small lymphocytes in affected paracortical lymphoid tissue, transitional zones, and medullary areas. Lymphoblasts were characterized ultrastructurally by large pale nuclei, increased cytoplasmic areas, few mitochondria, many cytoplasmic ribosomes (but little endoplasmic reticulum), and increased numbers of mitotic figures. Evidence of cell death (small irregular nuclei with marginated chromatin but few changes in cytoplasmic organelles) was common in large lymphocytes.

Foci of vasculitis, edema, and fibrin that effaced lymph node architecture were found in both groups at PID 3 but were most severe in 16M-infected goats, in which focal areas of exudation had expanded into large multifocal areas of tissue destruction throughout the deep cortex. Deep cortical areas including the transition zones had severe vascular and exudative reactions. Hemorrhage was present in the cords adjacent to the medullary zones. The most severe lymphocyte depletion occurred in areas with severe vascular reactions.

Recovery of lymph node architecture at PID 28 and beyond was accompanied by marked medullary plasmacytosis, repopulation of both cortical and medullary areas by lymphocytes, and re-formation of germinal centers. The marked increase in germinal centers which occurred in the deep cortex and medullary marginal zones included a spectrum from small foci of blast cells to large clearly defined mature germinal centers with large numbers of mitotic figures and a corona of collagen.

Brucellar antigens stained for immunoperoxidase reactivity were found only in samples taken at PID 3, 7, and 14. There was no detectable difference in antigen distribution between the two groups of infected goats. Stained areas in infected goats were most prominent in tissues affected by acute inflammation, especially in focal aggregates of macrophages, neutrophils, and necrotic tissue. Typically, areas of the superficial cortex with severe lymphocytolysis and blast cell proliferation did not contain immunoreactive antigens. At PID 28, all sections were negative for brucellar antigens. There was no staining for brucellar antigens in any control tissues.

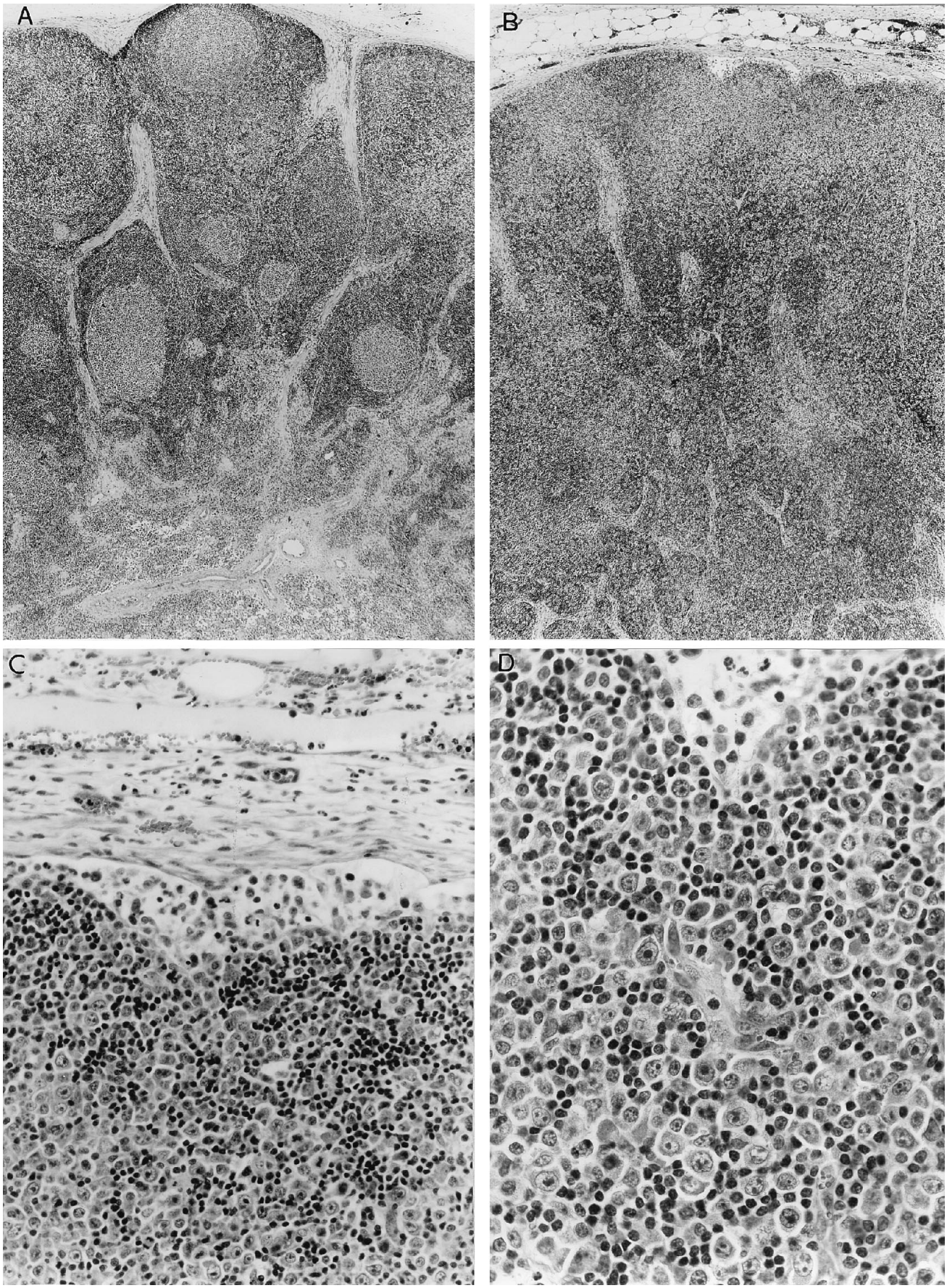
Bacteria were specifically labeled with the immunogold staining technique. Gold-labeled bacterial surfaces were identified in lymph node tissue by electron microscopy in samples taken at PID 3, 7, and 14 from both 16M- and Δ*purE201*-infected goats. Ultrastructurally, bacterial cells were found only intracellularly and only in macrophages, small lymphocytes, and neutrophils. In capsular foci of inflammation, bacterial cells were readily identified, most often in macrophages with ultrastructural evidence of activation. In contrast, bacteria were difficult to locate in lymph node cortices, even in sections with severe lesions taken at PID 3.

Analysis of 300 cortical cells that contained bacteria showed that 97% were macrophages, and 3% were small lymphocytes. In macrophages, bacteria were found only in phagolysosomes which typically contained degraded bacterial ghosts and bacterial remnants (Fig. 6). Bacteria in small lymphocytes were found only in phagosomes; the bacterial cell was intact, and there was no evidence in the phagosomes of dense granular material or vacuoles that suggested lysosomal fusion. Ultrastructural changes in degenerate small lymphocytes were cell swelling and nuclear chromatolysis (Fig. 7).

DISCUSSION

B. melitensis Δ*purE201* may be useful as a vaccine. It established infection in goat lymph nodes and was cleared from host tissue at approximately the same rate as its virulent parent, 16M. In contrast to 16M-infected goats, Δ*purE201*-infected goats did not have bacteria in tissues at necropsy and had less marked pathologic changes in regional lymph nodes. Possibly the *purE* mutant incites less inflammation yet replicates in lymphoid tissues and causes lymphocyte death like the more virulent parental strain 16M because of a loss of the capacity to damage vascular endothelium. There may be loss of a surface component that is required to initiate the inflammatory response; e.g., *B. abortus* RB51, a low-virulence mutant, repli-

FIG. 4. Superficial cervical lymph nodes, biopsy PID 3. (A) Control (goat 13). Cortex has many lymphocytes and large germinal centers but few cells in medulla; there is clear demarcation of cortex and medulla. (B) Δ*purE201* group (goat 7). There is acute serous lymphadenitis with diffuse depletion of cortical lymphocytes, loss of germinal centers, hypercellular medulla, and capsulitis (top). (C and D) Enlargements of panel B. (C) Capsulitis, dilatation of afferent lymphatics and capsular veins, filling of marginal sinuses with inflammatory cells, loss or pyknosis of cortical small lymphocytes, and increase of large blast cells. (D) Large blast cells are cuboidal or stellate shaped and have large nuclei and nucleoli.


A
B
C
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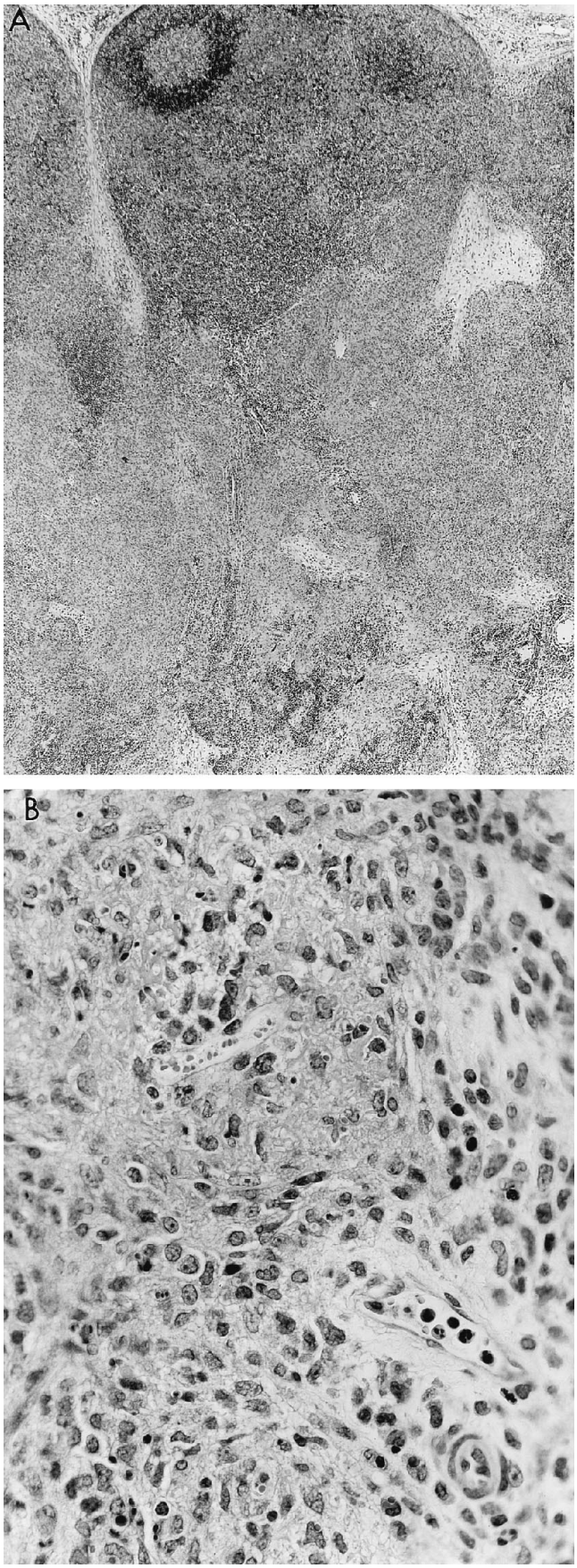

cates in the placenta like its virulent parent but, unlike the virulent strains, does not induce abortion because its lipopolysaccharides lack O side chains (4).

Serologic titers to both mutant and parental strains were similar to those seen in field vaccination of goats with standard doses of *B. melitensis* Rev 1 vaccine (8, 24, 30). The relatively low IgG dot blot response to whole cells compared with a similar response (presumable IgM) in the tube agglutination test may reflect reduced severity of infection. In humans, IgG rather than IgM titers in the agglutination test correlate with disease activity, and a rise in IgG titer may indicate a relapse of disease. The difference in IgG titer between Δ*purE201*- and 16M-vaccinated groups may not indicate differences in protection in vivo since phagocytosis of *B. abortus* is not sufficient to prevent infection (6, 26). Passive transfer of *Brucella*-specific antibodies to mice briefly reduces but does not prevent spleen colonization following challenge (5, 23). Others have reported that induction of specific cell-mediated immunity is required for clearance of *B. abortus* (2, 3). In our study, lymphocyte blastogenesis assays revealed similar responses by both vaccine groups with cell-mediated responses present at 12 weeks after infection.

The *purE* mutant survived and replicated in lymphoid tissue at titers like those of the virulent *B. melitensis* 16M, a major benefit in its usefulness as a vaccine. The large doses used and the diffuse lymphadenitis and lymphocyte destruction that occurred in the regional lymph node may have obscured significant differences in bacterial persistence between the two strains.

Levels of all bacterial CFU were higher in the first week, with a gradual decline thereafter. There may have been killing and replication occurring simultaneously after the first week or, alternatively, no further bacterial replication, with bacteria gradually being killed by host immune responses. In either case, it is possible that the intense inflammation caused by the inoculation of large numbers of bacteria resulted in delivery of purines to intracellular bacteria. On the other hand, the failure of Δ*purE201* to be killed more rapidly after inflammation (and purine delivery) has subsided suggests that deletion of the *purE* gene may not impair the ability of *B. melitensis* to resist intracellular killing in goats.

The failure of Δ*purE201* to be cleared more rapidly than 16M in this goat model is intriguing. Mice clear the identical *purE* mutant strain from their spleens in only 8 weeks but remain infected with 16M for at least 3 months (6a, 6b). The attenuating effects of *pur* mutations cannot be readily predicted and vary with the bacterial species, challenge host, and location of the mutation in the purine synthesis pathway. In general, mutations in genes encoding early steps in the pathway (before synthesis of IMP), like that contained in Δ*purE201*, are less attenuating than mutations (e.g., in *purA*) after IMP synthesis. For example, mutation of *purE* in *Salmonella typhimurium* attenuates mouse virulence approximately 3 logs (19), but mutation in *purA* attenuates by at least 8 logs (22). Moreover, *Salmonella* strains deficient in *purA* are completely avirulent (and nonimmunogenic) in humans (18). Interestingly, an early purine pathway mutant of *Vibrio cholerae* that is attenu-

FIG. 5. Acute serous lymphadenitis with severe exudative inflammatory responses in lymph node, 16M-infected goat 1, PID 3. (A) Serofibrinous inflammatory reaction effaces the deep cortex and medullary zones of the lymph node. (B) Exudates of fibrin and albumin surrounding postcapillary venules and neutrophils in the lumen on one venule. There are severe vasculitis, hemorrhage, and fibrin deposition.

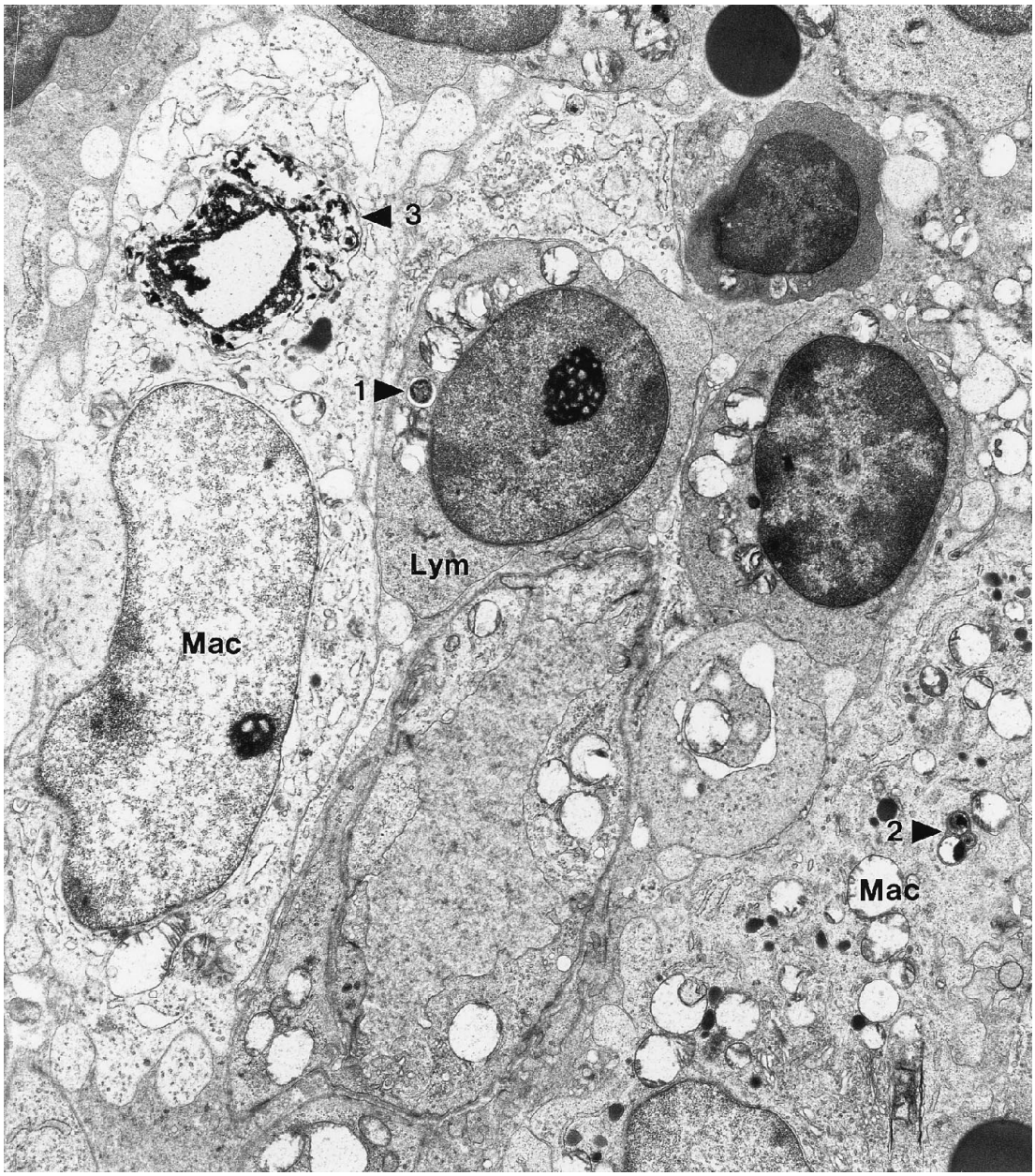

FIG. 6. Ultrastructure of lymph node cortex, 16M-infected goat, PID 3. Large pale macrophages (Mac) have phagolysosomes filled with bacilli in various stages of degradation (arrowhead labeled 2) or with electron-dense cellular debris and degenerate macrophages (arrowhead labeled 3). One lymphocyte (Lym) contains phagosome with an intact bacterium (arrowhead labeled 1). Macrophages have irregular surfaces, cytoplasmic vacuoles, and dilated rough endoplasmic reticulum. Mitochondria are swollen in all cells, endothelium is swollen, and there is loss of cytoplasmic detail but preservation of nuclear structures. Bar = 1.3 μm.

ated for mice is fully virulent in guinea pigs (10), while *purE* mutants of *Shigella flexneri* show little or no attenuation when tested by inoculation into guinea pig conjunctivae (11, 16). Guinea pig conjunctival fluid contains sufficient purines to support growth of the Δ*purE S. flexneri* (11), while mouse peritoneal fluid is devoid of purines (12). Thus, identical *pur* mutants may express different levels of attenuation in different challenge hosts. These differences in attenuation may depend, at least in part, on the levels of purines available in host tissues.

Lymphocyte death may result from a direct infection of lymphocytes, since bacteria were seen ultrastructurally in small lymphocytes. Alternately, death of lymphocytes may arise from an indirect effect of cytokines that arise from infection in adjacent areas. Supporting this hypothesis are the histochemical data that show intact bacteria and bacterial antigens outside the cortical zones of the lymph node and ultrastructural evidence which suggests that large lymphocytes die by a pattern of cell death referred to as apoptosis, a mechanism that involves tumor necrosis factor alpha and other potent macrophages cytokines. The destruction of lymphoid tissue by facultative intracellular bacteria is commonly associated with immunosuppression, and this may be a strategy of microbial pathogens to enhance infection of the animal host; e.g., infection with a virulent *Salmonella* strain can result in immunosuppression which in turn facilitates establishment of the carrier state (7, 18, 25).

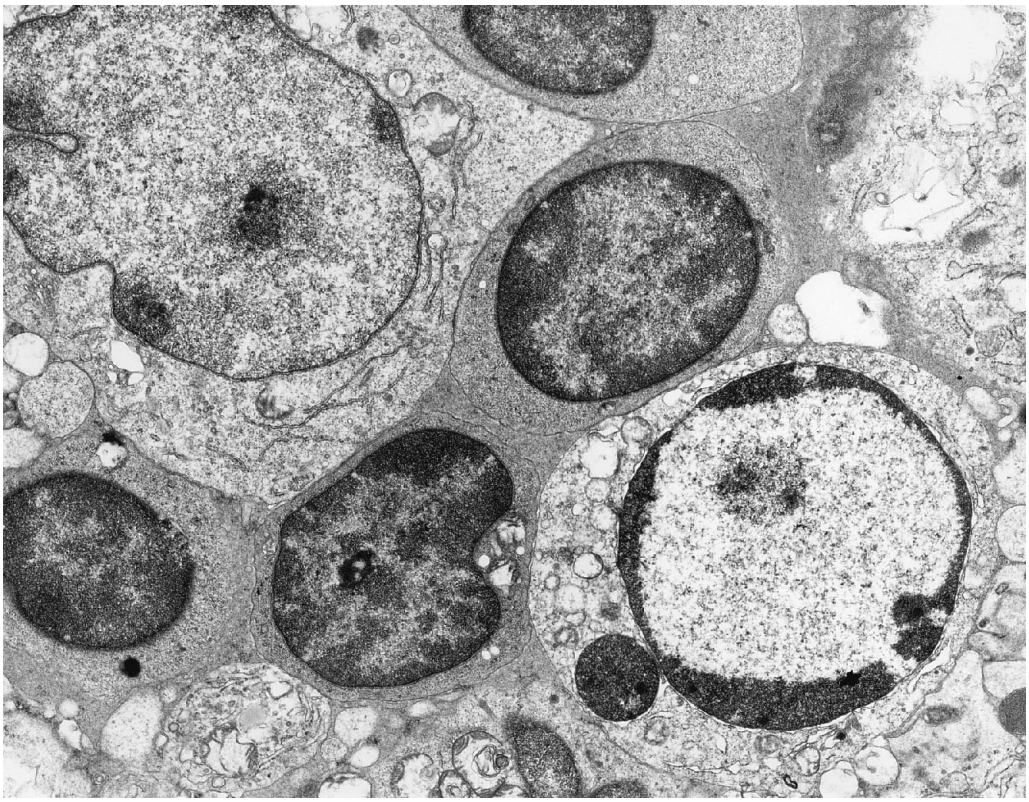

FIG. 7. Superficial cortex of lymph node, Δ*purE201* mutant-infected goat 8, PID 7. At the center are small dark lymphocytes. A large transitional blast cell (upper left) has a pale nucleus with a large nucleolus, many polyribosomes but little endoplasmic reticulum, Golgi complex with few vacuoles and poorly developed trans-Golgi area, and few mitochondria. A large lymphocyte (lower right) is spherical, has marginated chromatin typical of apoptosis, and has other evidence of degeneration, including cytoplasmic dense bodies, dissolution of the nucleolus, and mitochondrial swelling with cristolysis and membrane aggregation. Bar = 1.3 μm.

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